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FLY AND CRAYFISH AND THE
EFFECT OF DDT

A DISSERTATION SUBMITTED TO THE FACULTY OF THE
DIVISION OF THE BIOLOGICAL SCIENCES IN CANDI-
DACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY
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ACETYLCHOLINE AND RELATED SUBSTANCES IN THE COCKROACH, FLY AND CRAYFISH AND THE EFFECT OF DDT¹

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With emphasis on cockroach nerve cord and the effects of DDT, acetylcholine content and distribution, the effect of administered choline esters and related pharmacological agents, esterase activity, ratio of free acetylcholine to bound precursor, and acetylcholine synthesis have been studied. The fly, crayfish, frog and rat have also been used, though less extensively.

The work was stimulated by an interest in the mechanism of action of DDT in insects. Parallel research has considered quantity required to kill and absorption through the chitinous exoskeleton (Savit, Kollros and Tobias, '46; Tobias, Kollros and Savit, '46; Richards and Cutkomp, '46), loci of action (Yeager and Munson, '45; Roeder and Weiant, '46; Tobias and Kollros, '46) and generalized biochemical effects (Tobias, Merrill and Savit, '46). Since the neuromuscular apparatus is clearly involved, it seemed that valuable insight might be gained if some measurable biochemical change in that system could be followed.

It was suggested that DDT might lower synaptic resistance through cholinesterase inhibition (Metcalf and Kearns, '45). The suggestion stemmed from a resemblance of the symptoms of DDT poisoning in the roach to those following eserine injection, and from a potentiation of DDT toxicity by eserine.

¹ DDT, 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane.

² This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

No inhibition of esterase activity could be found (Horecker and Sambursky, '45; Richards and Cutkomp, '45), but it was still possible that DDT might increase acetylcholine by accelerating synthesis or by liberating free ester from bound precursor, rather than by inhibiting destruction.

It was first sought to establish whether DDT altered nervous system acetylcholine, and secondly, to investigate mechanisms by which the change might be effected.

METHODS

The cockroach (*Periplaneta americana*), fly (*Musca domestica*), crayfish (*Cambarus* spp.), frog (*Rana pipiens*) and white rat were used. Roaches were poisoned by 5–10 minutes confinement in a DDT coated glass cylinder; flies by direct body surface application of 20 mg of DDT per kg (Savit, Kollros and Tobias, '46; Tobias, Kollros and Savit, '46). Crayfish were given 30 mg of DDT per kg, intraabdominally in emulsion (1% DDT, 1% lecithin, 10% peanut oil, 88% insect Ringer (Phillips and Gilman, '46). Frogs received 100 mg per kg in emulsion in the dorsal lymph sac, and rats were given 800–1600 mg per kg orally in peanut oil or intraperitoneally in emulsion.

In the roach, the individual nerve cord (thoracic ganglia and connectives, 3–5 mg) was assayed. The insect was pinned on its back, decapitated, and the legs were amputated. The thoracic cord was exposed by removing the covering exoskeleton. The post-metathoracic connectives, held in a jeweler's forceps, were cut with iris scissors, and the animal was flooded with eserinated Ringer's solution (NaCl 0.67 gm, KCl 0.015 gm, CaCl_2 0.012 gm, NaHCO_3 0.015 gm, eserine salicylate 10 mg, H_2O to 100 cm^3). The cord was then dissected out anteriorly, removed, blotted, weighed on a torsion microbalance (sensitivity 2 γ), homogenized in a small glass homogenizer (capacity about 0.75 cm^3) containing 0.05 cm^3 of solution, diluted to a total of 0.2 cm^3 per mg, and assayed, without centrifuging, on the eserinated, frog, rectus

abdominis for so-called "free" acetylcholine. All assays were made in a 4 cm³ muscle chamber at 6 minutes after cutting the caudal connectives, constancy of timing being important for reproducibility (Tobias and Lepinat, '46). With some modification of the dissection, crayfish nerve cord (thoracic and abdominal) was similarly handled.

Total acetylcholine was assayed by several methods. When an aliquot was removed for free ester assay, the remaining suspension was brought to pH 3 to 4 with 0.5 N HCl. This was then either left for 1 hour at room temperature or was put in a boiling water bath for 2 minutes and subsequently cooled. In either case, the solution was finally neutralized with 0.5 N NaOH, and assayed for total acetylcholine. Liberation of free from bound acetylcholine with excess K⁺ (Mann, Tennenbaum and Quastel, '38; Schallek, '45) is described below.

In the case of the fly, the whole animal was ground (Potter-Elvehjem homogenizer), diluted to the same extent as roach cord and centrifuged. An aliquot of supernatant was then assayed as already described. Frog and rat brain were assayed as described elsewhere (Tobias, Lipton and Lepinat, '46).

Cholinesterase activity was determined in roach cord only. Cords were exposed, left in situ and kept moist with insect Ringer's fluid. When a sufficient number had been prepared, they were excised, blotted, weighed, pooled in groups of three to five and homogenized, in the small homogenizer described above, with a solution containing NaCl 0.15 M, MgCl₂ 0.04 M and NaHCO₃ 0.025 M. Esterase activity of this homogenate was determined in the Warburg. The suspension was tipped into an acetylcholine or acetyl- β -methylcholine solution and CO₂ evolution was measured at 37°C. Final concentrations in the reaction mixture were: ester 0.01 M, cord tissue 3.3 mg per cm³.

Details of synthesis experiments are described below.

RESULTS

Central nervous system acetylcholine

It is clear (table 1) that after DDT poisoning, central nervous system acetylcholine rises strikingly, but only in some species and then only under certain conditions.

Normal roach cord contains about 33 γ of free acetylcholine per gram, fifteen times the average amount in mammalian, central neural tissue (Feldberg, '45); that of the 17–24 hour poisoned, prostrate roach about 102 γ per gram, an increase of 210%. The whole, normal fly (wings and legs amputated) contains some 47 γ per gram, the prostrate, nearly motionless DDT-poisoned fly 131 γ per gram, an increase of 180%. That this increase was, in reality, in the central nervous system in the fly also was shown by the fact that the head and thorax, which contain the largest mass of neural tissue, contained 140 γ of acetylcholine per gram as compared to 11 γ per gram in the abdomen. This rise following DDT has been confirmed by others for the meal worm (*Tenebrio molitor*) and the grasshopper (*Melanoplus differentialis*) (Barron and Lipton, '45). In the cockroach and fly, total acetylcholine also increased some 113% and 215% respectively (table 1). In the crayfish, free, cord acetylcholine rose from 28 γ per gram in the normal to 38 γ per gram in the DDT-poisoned, prostrate animal—a small change compared to that in the cockroach and fly. No acetylcholine change was found after DDT, in frog brain, rat brain or rat salivary gland.

There seem to be two prerequisites for the acetylcholine increase to occur after DDT. Prostration must occur and sufficient time must elapse. No rise is detectable in the roach and it is relatively small in the fly until symptoms progress to the prostrate, practically immobile stage. Poisoned roaches which do not reach this stage or which are still markedly tremulous show practically no increase in cord acetylcholine. Secondly, rapid induction of prostration by injection of an excessive dose of emulsified DDT is not accompanied by a

TABLE 1
Nervous system acetylcholine content, normal and after DDT

ANIMAL AND TISSUE	HOURS AFTER DDT ¹	SYMPTOMS WHEN ASSAYED	ACETYLCHOLINE, γ/gm ²		
			No.	Free	Total
Cockroach (<i>Periplaneta americana</i>), thoracic nerve cord	..	normal controls	28	34 ± 0.88	20
	6	none, tremulous or hyperactive	3	41	46 ± 1.5 ³
	20	tremulous	3	41	
	20	prostrate, nearly motionless	10	102 ± 8.1	3
Fly (<i>Musca domestica</i>), whole body	..	normal controls	3	47	3
	2	tremulous and hyperactive	2	61	1
	4	prostrate, nearly motionless	2	131	2
					148
Crayfish (<i>Cambarus</i> spp.), thoracic nerve cord	..	normal controls	9	28 ± 1.6	3
	7	prostrate, slightly active	4	33 ± 5.1	1
	20	prostrate, nearly motionless	6	38 ± 1.4	2
					148
Frog (<i>Rana pipiens</i>), brain	..	normal controls	6	2.1 ± 0.13	6
	20	prostrate, nearly motionless	6	2.1 ± 0.05	6
					4.1 ± 0.36
					4.6 ± 0.40
Rat, brain	..	normal controls	3	1.4	3
	4	tremulous or grossly convulsive	3	1.3	3
					4.2
					3.9
Rat, submaxillary salivary glands	..	normal controls	6	1.4 ± 0.17	6
	4	tremulous or grossly hyperactive	7	1.2 ± 0.05	7
					$2.25 - 0.20$
					$2.57 - 0.15$

¹ DDT given as indicated under "Methods."

² Reliability of data expressed as $\pm$ S.E.

³ Includes assay for total ACh by all methods used. For breakdown see table 4.

rise in cord acetylcholine. Apparently considerable time must elapse. The elevated cord acetylcholine level is therefore associated with the prostrate phase of the poisoning and dependent upon the passage of a given amount of time. The fact that both prostration and acetylcholine increase appear earlier in the fly than the roach fits the finding that an LD-50 dose of DDT kills the fly much more rapidly (hours) than it does the roach (days) (Tobias, Kollros and Savit, '46).

It is of interest that elevated cord acetylcholine is associated with the phase of inactivity and not with that of earlier hyperactivity. This is reminiscent of a rise in frog and rat brain acetylcholine after chloroform or nembutal, but not during the convulsions following strychnine or picrotoxin (Tobias, Lipton and Lepinat, '46).

Why the rise is so small in the crayfish, and undetectable in the frog and rat after DDT is not known. The rat exhibits no symptoms comparable to the prostrate immobility of the insect. It dies during a convulsion or recovers. Presumably roaches can survive complete prostration and immobility because diffusion provides adequate respiratory exchange (Wigglesworth). It was therefore hoped that the frog, which survives mechanical respiratory arrest because of skin respiration, would become prostrate and immobile as does the roach. This did occur, but no rise in brain acetylcholine was found.

Since there was no detectable effect of DDT on acetylcholine in rat brain, it seemed useful to examine some cholinergic effector. Therefore, the free and bound acetylcholine content of the rat submaxillary salivary gland was measured. The data are shown in table 1; it can be seen that DDT produced no significant change.

Distribution of acetylcholine in ganglia and connectives

It was of interest to know whether roach cord acetylcholine was increased to the same extent in the cellular ganglia as in the primarily fiber containing connectives. The data are

shown in table 2. In the normal roach and crayfish, free acetylcholine is seen to have been about 1.7 times as concentrated in the ganglia (58 and 36 γ per gm) as in the connectives (34 and 21 γ per gm). After poisoning with DDT, however, practically the entire acetylcholine rise, in the roach, was in the connectives. In the crayfish, the findings are similar though not so obvious, in keeping perhaps with the smaller total rise in the crayfish cord than in the cockroach.

*Partial identification of active substance
as acetylcholine*

Since various substances can cause the assay muscle to contract, it was necessary to demonstrate that the active material was acetylcholine. Chemical identification was not attempted. Cord extract from normal or poisoned animals, however, was inactive if prepared without eserine, and lost its activity when heated (2 minutes in boiling water bath) in alkaline solution. Nor did cord extract have any effect on the curarized frog rectus. These findings are in keeping with the assumption that the active substance is acetylcholine (Gaddum, '36; Kuffler, '43).

It could be argued that residual DDT or derivatives of it in cord extract might be responsible for the increased assay muscle contraction. Analysis of roaches most recently poisoned does not indicate excess acetylcholine, however, and DDT emulsion added directly to the water bath evokes no contraction by the assay muscle. Therefore, DDT is eliminated as being responsible for the increased assay muscle response. Nor did any contraction occur when DDA ($-\text{COOH}$ substituted for $-\text{CCl}_3$ in DDT), a possible intermediary metabolite of DDT (Stohlman and Smith, '45) was added.

Water content of nerve cord

Dissection of the prostrate roach gives an impression of dehydration and loss of fat from the fat body. Objective measurements of the whole animal have not shown any sig-

TABLE 2
Acetylcholine content of ganglia and connectives, normal and after DDT.

ANIMAL	HOURS AFTER DDT	SYMPTOMS WHEN ASSAYED	FREE ACETYLCHOLINE, γ /GM		AVERAGE RATIO, CONNECTIVES GANGLIA
			ganglia	connectives	
Cockroach (<i>Periplaneta americana</i>)	..	normal controls	62, 52, 60	39, 33, 29	0.58
	21	prostrate, slight twitching	59, 95, 67	66, 98, 97	1.20
	72	prostrate, nearly motionless	58	134	2.32
Crayfish (<i>Cambarus</i> spp.)	..	normal controls	20, 35, 45, 52	19, 18, 19, 17	0.63
			37, 45, 31, 34	17, 18, 25, 30	
			26	22	
	7	prostrate, slight twitching	31, 47, 53, 41	13, 17, 33, 31	0.54
	20	prostrate, nearly motionless	43, 34, 29, 63	50, 40, 22, 47	0.98
			29, 35	32, 33	

Literature values, normals

ANIMAL	ACETYLCHOLINE, γ /GM		RATIO, CONNECTIVES GANGLIA
	ganglia	connectives	
Cambarus, fall (Smith, '39)	14	4	0.29
Cambarus, spring (Smith, '39)	46	13	0.28
Homarus, spring (Smith, '39)	17	9	0.53
Carcinus (Richards and Cutkomp, '46)	100	2	0.02

nificant change in per cent water content after poisoning (Tobias, Merrill and Savit, '46). Still it was necessary to measure water content of normal and poisoned roach cord, since local dehydration might account for some small part of the apparent acetylcholine rise. Thoracic nerve cord was therefore removed, blotted, weighed with a torsion microbalance (maximum load 5 mg, sensitivity 2 γ) on an aluminum foil dish (1 to 1.5 mg), dried at 105°C. for 48 hours and reweighed. Ten normal and ten poisoned insects were used.

The normal cord was found to be $73.9 \pm 0.95\%$ ³ water; the poisoned was $75.3 \pm 1.56\%$. This difference is not significant, and there is no evidence of cord dehydration.

Nerve cord esterase activity

Normally active cholinesterase is found in whole, poisoned roach brei (Horecker and Sambursky, '45) and DDT does not produce inhibition of cholinesterase when added to roach cord in vitro (Richards and Cutkomp, '45). This failure of DDT to inhibit in vitro is, however, of questionable significance, because of the extreme insolubility of DDT in water. Primarily because cord acetylcholine was found to be elevated after DDT only under the conditions noted above, esterase measurements were made at several times after DDT administration, including that at which ester content was known to be elevated. Because roach cord had been shown to have considerably more esterase activity for acetyl- β -methylcholine than for acetylcholine (Richards and Cutkomp, '45) activity was measured for both substrates.

The results are shown in table 3. The QChE of normal roach cord is seen to be about thirty-two for acetylcholine and 52 for acetyl- β -methylcholine. There was no significant change after poisoning.

Free and bound acetylcholine in nerve cord

Free acetylcholine is generally thought of as that fraction of the ester readily extractable by simple homogenization with

³ Given as $\pm$ S.E.

eserinized Ringer solution. Bound acetylcholine, however, is presumably not so easily extractable. More firmly held to some tissue constituent, it requires liberation by more drastic means, such as heating (pH 3 to 4 to avoid acetylcholine destruction), standing for some time in acid (an hour at pH 3 to 4 at room temperature) (Mann, Tennenbaum and Quastel, '38), extraction by a solution rich in potassium ions (Schallek, '45), or one containing chloroform. Because of the suggestion that DDT might act by liberating free acetyl-

TABLE 3
Cholinesterase activity of ventral nerve cord of the cockroach.

Normal and after DDT.		
NO. AND CONDITION OF ANIMALS	SUBSTRATE	ESTERASE ACTIVITY qChE ¹
4 normals	acetylcholine	31
4 normals	acetylcholine	33
2 normals	acetyl- β -methylcholine	52
DDT poisoned		
4 hyperactive	acetylcholine	38
4 prostrate	acetylcholine	36
4 prostrate	acetylcholine	32
4 nearly motionless	acetylcholine	35
2 hyperactive	acetyl- β -methylcholine	50
2 prostrate, nearly motionless	acetyl- β -methylcholine	52

¹QChE = mg of substrate split by 100 mg of fresh tissue in 60 minutes. Given here at 37.6°C.

choline from so-called precursor, the ratio of free to bound ester was measured in normal and poisoned roach cord.

It has been reported that the nerve cords of the roach, lobster and other arthropods contain no bound acetylcholine (Corteggiani and Serfaty, '39). More recent studies on lobster cord have, however, demonstrated bound acetylcholine by several methods, including extraction with a K⁺ rich solution (Schallek, '45). For roach cord, several extraction techniques have been employed; those involving heat and acidification are described in the section under methods. Liberation with

excess potassium simply involves extraction of homogenized cord with 0.5 M KCl in modified Ringer, at room temperature. The excess K^+ must be sufficiently diluted before the solution contacts the assay muscle. Such extraction for 6 minutes (table 4) does not liberate any bound ester; 1 hour does, but 2 hours extraction does not further increase the yield.

It is clear (tables 1 and 4) that, on the average, about 34% of normal roach cord acetylcholine is in the bound form (compare rat brain, 80%) (Tobias, Lipton and Lepinat, '46). In the DDT prostrate animal, however, it is either all in the free form, or the bound fraction becomes a very much smaller part of the total than in the normal insect. The interpretation of this finding will be considered below. Attempts have also been made to measure in vitro liberation of free ester from precursor in roach cord and rat brain. Normal nerve cords were removed and eserinated as described previously. In one case homogenization was carried out in the presence of added DDT (10 mg of DDT per gram of tissue; approximately 1000 times the LD-50 for the roach) (Tobias, Kollros and Savit, '46), in emulsion, and in the other, in the presence of an equivalent amount of emulsion, but without DDT. Each homogenate was assayed 5, 35, 75 and 120 minutes after mixing. Neither ever contained more than 40 γ of acetylcholine per gram of tissue, approximately the amount of free ester usually found in normal cord. A similar test (three experiments) was also negative with rat brain and has been confirmed by others (Welsh, '46). It has been claimed that still larger amounts of DDT can liberate free ester from precursor in vitro (Barron and Lipton, '45).

Synthesis of acetylcholine by nerve cord

Experiments have been done to determine whether DDT accelerates acetylcholine synthesis by roach cord in vitro, aerobically or anaerobically. The data are shown in table 5 with the composition of the medium used. A 3-hour period

was allowed for aerobic synthesis. Preliminary trials, however, showed that anaerobic synthesis was well advanced in 30 minutes, so this period was used. Roach cords were pooled in groups of 4 or 5, homogenized and added to the synthesis medium as indicated in table 5.

TABLE 5
Synthesis of acetylcholine by roach cord.

INSECT GROUP	AEROBIC OF ANAEROBIC	γ ACETYLCHOLINE SYNTHESIZED	
		per gm in 3 hrs.	per gm in 30 mins.
Normal controls ¹	Aerobic	9	
DDT prostrates ¹	'	6	
Normal controls ²		24	
DDT prostrates ²		24	
Normal controls ³	Anaerobic	..	47 (aver. of 6)
DDT hyperactive ³		..	47 (aver. of 2)
DDT prostrate ³ but moving		..	50 (aver. of 3)
DDT prostrate ³ and practically motionless		..	49 (aver. of 6)

Synthetic mixtures: All in phosphate buffer at 7.2. All experiments at 25 to 26°C.

¹ Lactate 0.01 M, KCl 0.05 M, NaCl 0.016 M, phosphate 0.005 M, eserine salicylate 2×10^{-4} M, choline chloride 0.003 M, sodium acetate 0.003 M, nerve cord 12 mg.

² As above plus glucose 0.01 M.

³ Choline chloride 1.04×10^{-3} M, NaF 2.09×10^{-2} M, KCl 1.74×10^{-2} M, eserine salicylate 8.7×10^{-6} M, ATP 1.6×10^{-3} M, yeast juice ⁴ 0.3 cm³, nerve cord 10.5 mg, frog Ringer up to a final volume of 2.9 cm³.

⁴ Yeast juice prepared by heating 1 gm dry yeast in 4 cm³ H₂O, centrifuging and neutralizing.

Apparently normal roach cord, under these experimental conditions, aerobically synthesizes about 2.7 times as much acetylcholine when supplied with both lactate and glucose (24 γ per gram in 3 hours) as with lactate alone (9 γ per gram). In either case, however, the cord from poisoned roaches synthesized no more ester than the normal. Anaerobically, normal cord synthesized 47 γ per gram in 30 minutes; tissue from poisoned animals synthesized 49–50 γ per gram, again, not significantly different from the normal. DDA

(Stohlman and Smith, '45), a possible metabolic intermediary, did not, in one experiment, accelerate synthesis when added to normal cord homogenate.

*Effect of various substances on nerve cord
acetylcholine and cholinesterase*

The findings discussed to this point raised the question of whether the change in cord acetylcholine after DDT was specific for that substance or would occur after almost any poisoning involving a period of prolonged prostration and immobility preceding death. Therefore, a variety of conditions was tried. The data are summarized in table 6.

Both eserine and barbital are seen to have caused a rise in cord acetylcholine. This is understandable on the basis of esterase inhibition; 100 mg of eserine per kg produced about 44% inhibition in 4 hours. Barbital, whose anticholinesterase activity in higher animals is still a matter of question (Schütz, '43) also decreased cholinesterase activity of roach cord. When acetylcholine content was elevated after barbital it was almost all in the free state (compare DDT). Complete deprivation of food and water for 3 days (poisoned roaches soon become unable to feed or drink) had no great effect on cord acetylcholine though it may have lowered it somewhat; certainly it caused no rise. Nor was a significant rise caused by 4 to 24 hours prostration after CO₂, insulin given in very large doses (8000 U. per kg) or injected nicotine (5 to 10 γ). On the other hand, cyclopropane as used for prolonged anesthesia (Tobias, Merrill and Savit, '46) or contact poisoning with hexachlorocyclohexane (Savit, Kollros and Tobias, '46) did raise cord acetylcholine content. The increase occurred more erratically than after DDT, but did occur often enough to be significant and interesting.

It is clear then that prolonged pre-mortem prostration need not be accompanied by a rise in cord acetylcholine. On the other hand, aside from known anticholinesterases, DDT is not the only substance which can produce a significant rise in roach cord acetylcholine content. A common basic mechanism will be sought.

TABLE 6
*Effect of various substances other than DDT on roach nerve cord
 acetylcholine content.*

EXPERIMENTAL CONDITIONS ¹	SYMPTOMS AND DURATION	ACETYLCHOLINE CONTENT, γ /GM	
		Individual	Average
Normal controls		46, 43, 29, 32	38
No food or water for 3 days	appear normal	26, 29	28
Intermittent CO ₂ inhalation	prostrate for 6 hours	40, 38, 30, 40	37
Insulin, 8000 U./kg	prostrate 24 hours	35, 40	38
Nicotine, 5 to 10	prostate 4 to 24 hours	38, 31, 40, 40, 40	38
Cyclopropane	12 to 18 hours anesthesia	44, 54, 40, 52, 60, 68, 124 96, 88, 126, 80, 96	77
Hexachlorocyclohexane (γ 666)	prostrate 20 hours	32, 29, 54, 56, 74, 56, 76, 76	57
Barbital, 2 gm/kg	prostrate 22 hours	52, 49, 66, 64, 64, 32, 46	53
Eserine, 2 mg/kg	no symptoms, 1 to 4 hours	43, 48	46
Eserine, 10 mg/kg	slight hyperactivity, 2 hours	49	49
Eserine, 25 mg/kg	prostrate, legs moving, 2 hours	48	48
Eserine, 25 mg/kg	prostrate, motionless, 7 hours	66	66
Eserine, 50 mg/kg	prostrate, slight activity, 2 hours	51	51
Eserine, 100 mg/kg	prostrate, slight activity, 2 hours	59	59
Eserine, 100 mg/kg	prostrate, slight activity, 4 hours	72	72
Eserine, 100 mg/kg	prostrate, nearly motionless 20 hours	140	140

¹ All substances except CO₂ injected intraabdominally.

Observations on administration of various choline esters, nicotine, eserine and atropine to the cockroach

To gain more insight into the function of acetylcholine in the roach, the effects of administering various choline esters and pharmacologically related substances were studied.

Acetyl- β -methylcholine, for which roach cord esterase activity is high (table 3), had no visible effect on normal roaches, even if injected in quantities as large as 20 grams per kg. Carbaminoylcholine, however, for which there is no known esterase, in doses of 1 gram per kg, caused almost immediate incoordinated hyperextension of the legs, ataxia and falling. The animals then lay prostrate, showing only occasional movement of legs, palpi and mouth parts, and died in a few hours. Doses of 0.5 and 0.1 gram per kg were ineffective. Acetylcholine, for which the roach cord possesses esterase activity about half as great as that for acetyl- β -methylcholine, might then be expected to be more effective than acetyl- β -methylcholine but less effective than carbaminoylcholine, and so it is. The injection of 20 grams per kg caused a single, short convulsive seizure, hyperextension of the legs, ataxia and falling. Prostration and death occurred in about 2 hours. Injection of 7 or 10 grams per kg, while causing no immediate changes, still resulted in prostration and death in 2-3 hours; 5 grams or less had no noticeable effect.

These doses are very large compared to those which produce effects in other animals (Goodman and Gilman, '41); the LD-50 for subcutaneous injections in rats is 4 mg of carbaminoylcholine per kg (compare to 1000 mg per kg above), 250 mg of acetylcholine per kg (compare to 10,000 mg per kg above) and 750 mg of acetyl- β -methylcholine per kg (compare to more than 20,000 mg per kg above). It has also been found that very large amounts of acetylcholine must be applied to roach cord to alter electrical activity (Roeder and Roeder, '39). However, a parallelism between esterase activity toward a substance and required lethal dose is apparent.

It is difficult to relate these findings to the elevated cord acetylcholine after DDT. It may be pointed out that symptoms which occur after injection of these esters resemble those caused by DDT, but the whole sequence of events is markedly foreshortened. The same is true following the injection of eserine (*vide infra*). Rough calculations show that the effective dose of injected acetylcholine (7 mg per roach) is approximately 70 times the amount contained in the cord of the DDT prostrated roach (table 1). In spite of this factor of 70, the quantities may be comparable at the site of action, if one considers the slow circulation and inefficient distribution of injected material (Tobias, Merrill and Savit, '46), the thick connective tissue sheath around the ganglia and the high esterase activity of the cord (table 3).

Eserine injected into the normal roach, in doses as small as 10 mg per kg, induced almost immediate, high frequency trembling, ataxia and falling. The animals lay prostrate and resembled DDT poisoned roaches in many respects. This similarity of symptoms and a potentiation of DDT toxicity by eserine have been commented upon before (Metcalf and Kearns, '45).

All these substances (choline esters, atropine, nicotine, eserine) have been applied directly to exposed nerve cord with intact sheath. The results would likely be modified if the experiments were repeated after opening the sheath. Even so, rather striking results have been obtained. The effect of nicotine application, in a concentration which does not block peripheral nerve, is described in detail elsewhere (Tobias and Kollros, '46). It produces a short-lived burst of high frequency mechanical (Tobias and Kollros, '46) and electrical (Pringle, '39) activity, followed by complete quiescence. This resembles its effect on autonomic ganglia in higher animals (Langley and Dickinson, 1890) and on the central nervous system in the frog (Libet and Gerard, '38). Such nicotization of a ganglion can stop hyperactivity previously induced in the segmental legs by DDT (Tobias and Kollros, '46; Yeager and Munson, '45). Locally applied atropine (5×10^{-2} M)

has a similar effect. It has been suggested that nicotine or atropine can quiet the hyperactive roach after DDT (Metcalf and Kearns, '45), but we have not been able to confirm this observation for the whole roach, in spite of the fact that local ganglionic application quiets the segmental legs. Eserine (5×10^{-4} M) applied directly to the ganglion, like nicotine, produces a short-lived burst of high frequency activity, followed by complete quiescence. Application of sufficient amounts of acetylcholine, acetyl- β -methylcholine or carbaminoylcholine has the same effect as eserine.

Effect of thiamine and excess potassium on the toxicity of DDT

Thiamine may be liberated at active nerve endings (von Muralt, '43); if it interacts in any way with acetylcholine effects at the end organ, and if acetylcholine is, in any way, causally related to the symptoms of DDT poisoning, it might also modify DDT action. Roaches were therefore given intra-abdominally injected thiamine (5 mg per kg) daily for 3 days. On the third day they received an LD-50 dose of surface applied DDT (10 mg per kg) 30 minutes after the thiamine and a fourth dose of thiamine 3 hours later. Controls received injections of 0.9% sodium chloride solution. The thiamine had no effect on rate of development of symptoms, severity of symptoms or mortality.

Similarly, if acetylcholine is important in DDT poisoning, potassium might potentiate the effects of DDT. Therefore emulsified DDT was injected into cockroaches in normal Ringer's fluid and in Ringer's fluid containing five times the usual amount of potassium. Potassium did not potentiate the effect of DDT beyond its own toxicity.

DISCUSSION

Central nervous system acetylcholine content rises at certain times after DDT, most strikingly in the cockroach and fly (about 200%), minimally in the crayfish, but not in the rat or frog. In the roach and fly the high content is found during

the late period of prostration and relative immobility rather than during the early period of great hyperactivity. This is reminiscent of the rise in rat and frog brain acetylcholine after chloroform or nembutal, but not during the convulsions following strychnine or picrotoxin.

In the roach, the rise is apparently all in the connectives rather than in the ganglia, and a similar change occurs in the crayfish, though less strikingly. This is of interest because, to our knowledge, it constitutes the only condition, aside from K^+ administration, which increases acetylcholine content of a structure composed primarily of nerve fibers, *in vivo*, by a non-anticholinesterase. Liberation of acetylcholine *in vitro* from stimulated nerve fiber has been described (Lissak, '39), but this is quite a different matter.

The active substance satisfies the tests for acetylcholine to which it has been subjected. Since there is no change in cord water content, none of the acetylcholine rise is due to simple concentration. The effect is not unique for DDT; an acetylcholine increase is also produced by prolonged anesthesia with cyclopropane and by another insecticide, γ hexachlorocyclohexane.

One step in the mechanism of increase of acetylcholine in roach cord after DDT appears to be the liberation of free ester from bound precursor. In the normal roach cord, about 20% of the ester is in the bound form. When it increases after DDT, however, it is either all or almost all in the free form. It is suggested, therefore, that DDT, or more likely some metabolic intermediary, causes a liberation of free from bound ester. This might shift an equilibrium to favor formation of precursor. Such a new precursor could then be acted upon to liberate more free ester and so on. A reaction chain of this sort need not manifest itself *in vitro* possibly because of some deficiency in supplied substrates, catalysts, or the like. It has been suggested that DDT might most effectively act on such a precursor, if it is an acetylcholine-lipoprotein complex (Barron and Lipton, '45).

The original hypothesis then, that the poisoning might be accompanied by liberation of free acetylcholine from bound precursor has been largely validated for the roach. There is no explanation of why the rise is seen only during late prostration and not during the earlier hyperactivity; nor is it clear why the increase is found only in insects (cockroach, fly, grasshopper, meal-worm) and not in the frog or rat. Any relation which there may be between acetylcholine rise, any symptoms of DDT poisoning, or cause of death is therefore not apparent.

It is interesting that eserine, which inhibits cholinesterase in the roach as in other animals, produces symptoms in the roach which very much resemble those produced by DDT. This is true whether the eserine be given intraabdominally or by local application to one of the thoracic ganglia. This, and the fact that the hypermotor symptoms of DDT or eserine poisoning can be stopped by the application of nicotine or atropine to the ganglion, emphasizes the importance of synaptic structures in the maintenance and development of the early symptoms of hyperactivity after DDT.

The fact that it requires enormous quantities of intraabdominally injected carbaminoylcholine, acetylcholine or acetyl- β -methylcholine to produce symptoms in the roach may possibly be explained on the basis of its very slow delivery to neural structures, as a result of slow circulation and relative impermeability of the nerve cord sheath.

CONCLUSIONS

1. The thoracic portion of the cockroach (*Periplaneta americana*) ventral nerve cord normally contains about 33 γ of free and 12 γ of bound acetylcholine per gram, that of the crayfish (*Cambarus* spp.) about 28 γ of free ester per gram; the whole body of the fly (*Musca domestica*) contains about 47 γ of free ester per gram, almost all in the head and thorax.

2. During the late prostrate phase of DDT poisoning, but not during the early hyperactive phase, the free acetylcholine of the roach and fly central nervous systems rises about

200% ; the crayfish shows a much smaller rise; free and total acetylcholine content of rat and frog brain and of rat submaxillary gland are not changed.

3. In normal roach or crayfish cord the acetylcholine concentration is some 70% higher in the ganglia (58 and 36 γ per gram) than in the connectives (34 and 21 γ per gram); during prostration, in the roach after DDT, practically the entire rise in ester concentration is in the connectives; the change in the crayfish is similar though less obvious.

4. Esterase activity of normal roach cord is about 1.7 times as great for acetyl- β -methylcholine (QChE — 52) as for acetylcholine (QChE — 32), and neither is affected by poisoning with DDT.

5. In the media used, homogenized roach nerve cord synthesized 2.7 times as much acetylcholine aerobically with lactate-glucose substrate as with lactate alone. Anaerobically, synthesis of 47 γ per gram in 30 minutes was attained. DDT-poisoned cords did not synthesize acetylcholine any faster than did the normal.

6. Complete deprivation of food and water for 3 days, or prostration caused by CO₂, insulin or injected nicotine caused no rise of cord acetylcholine. Eserine and barbital did increase it but both showed anticholinesterase activity. Increases were also produced by cyclopropane or γ hexachlorocyclohexane.

7. Roaches tolerate very large doses of intraabdominally injected carbaminoylcholine, acetylcholine or acetyl- β -methylcholine, and the tolerated doses fall in the same order as the cord esterase activities for these esters. Nicotine, eserine or atropine applied to a nerve cord ganglion produce a short-lived burst of great hyperactivity and subsequent complete quiescence in the segmental legs. Each of them, locally applied, finally produces quiescence, even though there be pre-existent DDT hyperactivity. This emphasizes the importance of the ganglionic synapses in the hypermotor effects of DDT.

8. The active substance in roach and crayfish cord, which increases after DDT, satisfied those tests for acetylcholine to which it has been subjected.

9. The water content of the normal roach cord is 73.9%, and that of the prostrate roach after DDT is 75.3%. The acetylcholine increase is therefore, in no part, a simple concentration effect due to dehydration.

10. Bound ester is markedly decreased or entirely absent with a concomittant increase in the free fraction, when cord acetylcholine level is elevated.

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